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Transcriptional and Post-Transcriptional Regulation of Ribulose 1,5-Bisphosphate Carboxylase Gene Expression in Light- and Dark-Grown Amaranth Cotyledons

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The regulation of expression of the genes encoding the large subunit (LSU) and small subunit (SSU) of ribulose 1,5-bisphosphate carboxylase (RuBPCase) was examined in 1- through 8-day-old, dark-grown (etiolated) and light-grown amaranth cotyledons. RuBPCase specific activity in light-grown cotyledons increased during this 8-day period to a level 15-fold higher than in dark-grown cotyledons. Under both growth conditions, the accumulation of the LSU and SSU polypeptides was not coordinated. Initial detection of the SSU occurred 1 and 2 days after the appearance of the LSU in light- and dark-grown cotyledons, respectively. Furthermore, although the levels of the LSU were similar in both light- and dark-grown seedlings, the amount of the SSU followed clearly the changes in enzyme activity. Synthesis of these two polypeptides was dramatically different in etiolated versus light-grown cotyledons. In light the synthesis of both subunits was first observed on day 2 and continued throughout the growth of the cotyledons. In darkness the rate of synthesis of both subunits was much lower than in light and occurred only as a burst between days 2 and 5 after planting. However, mRNAs for both subunits were present in etiolated cotyledons at similar levels on days 4 through 7 (by Northern analysis) and were functional in vitro, despite their apparent inactivity in vivo after day 5. In addition, since both LSU and SSU mRNA levels were lower in dark- than in light-grown seedlings, our results indicate that both transcriptional and post-transcriptional controls modulate RuBPCase production in developing amaranth cotyledons.

Ribulose 1,5-bisphosphate carboxylase (RuBPCase) is located in the chloroplasts of all higher plants and is the primary enzyme of photosynthetic carbon fixation. This enzyme has a molecular weight of about 550,000 and consists of eight large (51- to 58-kilodalton [kDa]) and eight small (12- to 18-kDa) subunits (46), with the substrate binding site located on the large subunits (39, 40). The large subunit (LSU) is encoded on the chloroplast genome and is translated on 70S chloroplast ribosomes (19, 29). The small subunit (SSU) is encoded in the nucleus and is translated on free, cytoplasmic ribosomes as a 20-kDa precursor (14, 17, 22, 25, 32, 34). The precursor is processed to its final size during transport into the chloroplast, where it assembles with LSUs to form the active holoenzyme.

The gene for LSU is present in one copy per molecule of chloroplast DNA, and there may be up to several thousand copies of this DNA per cell (6, 52). In contrast, the SSU genes in all higher plant species examined occur as a small multigene family of 5 to 15 members (10, 13, 20, 27). Despite this disparity in gene copy number, approximately equal amounts of LSU and SSU mRNA and protein accumulate (24, 49, 50), suggesting coordinate expression of these chloroplastic and nuclear genes. In addition, studies with drugs which specifically inhibit chloroplastic or cytoplasmic protein synthesis have suggested a regulatory link between these two cellular compartments (4, 22, 28). However, the broad effects of these inhibitors make it difficult to interpret these studies.

Light is known to be involved in several aspects of the growth and development of plants. Light induces changes in the synthesis of a number of polypeptides, including the LSU and SSU of RuBPCase (3, 38, 54, 57, 58, 60). RuBPCase mRNA or protein accumulation (or both) have been shown to be strongly light dependent in peas (7, 21, 60, 64), soybeans (10) and *Lemna gibba* (61, 68). However, light-dependent production of RuBPCase is not universal, since in cucumbers (72) and maize (49), similar amounts of the enzyme are produced in dark- and light-grown plants.

Light-induced changes in SSU accumulation have been correlated with changes in levels of mRNAs which are translatable by using in vitro translation systems (55, 65, 68). Northern blot analysis with cloned SSU and LSU genes as probes has shown that light-stimulated RuBPCase accumulation in peas is accompanied by similar increases in mRNA levels for both types of subunit (9, 60). Furthermore, transcriptional-runoff experiments with isolated leaf nuclei have shown that the transcription of SSU genes in peas is light dependent (31). These studies suggest that the regulation of RuBPCase synthesis acts primarily at the level of transcription.

In addition to the control of RuBPCase expression by light which is observed in some plants, developmental regulation of RuBPCase levels has been shown to occur in the leaves of barley (50), wheat (24), and maize (43, 49) with increasing amounts of RuBPCase occurring from younger to older leaf regions. Thus, the control mechanisms of RuBPCase production may be complex, involving developmental and intercompartmental signals as well as light-mediated regulation. Moreover, significant variation in these controls is seen among different plant species.

We describe here the expression of RuBPCase LSU and SSU genes in a C_4-dicotyledonous plant, *Amaranthus hypochondriacus*. Although RuBPCase expression in a variety of plant species is being examined by other groups,

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amaranth is the only C_4 dicot currently under study. In view of the variations already observed among the different plant species, expression of the LSU and SSU genes in this unique plant group is of considerable interest. Changes in mRNA levels for each subunit were compared with changes in protein synthesis and accumulation in both light- and dark-grown amaranth cotyledons. Our results show that the degree of light-mediated RuBPCase gene expression observed was dependent on the time after planting at which the analysis was done as well as on the methods used for analyses. Furthermore, both transcriptional and post-transcriptional regulation modulated RuBPCase levels in developing amaranth cotyledons.

MATERIALS AND METHODS

Plant material and growth conditions. Seeds of *Amaranthus hypochondriacus* variety R103 were obtained from the Rodale Organic Gardening and Farming Research Center (36). Seeds were germinated, and plants were grown in a Conviron growth chamber at 24°C with 14-h/day illumination at an approximate intensity of 160 to 200 microeinsteins m^{-2} s^{-1}, and cotyledons were harvested at the appropriate time points. Dark-grown plants were germinated and grown in lightproof boxes which were placed in a dark room. Extreme care was taken during the growth of the dark-grown seedlings to avoid any exposure to light. Cotyledons from the dark-grown seedlings were rapidly harvested under a Kodak no. 7 green safelight and immediately frozen in liquid nitrogen or extracted.

RuBPCase assay. RuBPCase activity was determined as the RuBP-dependent, acid-stable radioactivity derived from $NaH^{14}CO_3$ (35). Protein was determined with a dye-binding assay (11).

Purification of RuBPCase and separation of LSU and SSU polypeptides. RuBPCase was purified from 100 to 150 g of mature amaranth leaves by the procedure of Sasaki et al. (55); in brief, fractionation was carried out by ammonium sulfate precipitation, gel filtration chromatography, and ion-exchange chromatography. Purification was monitored by specific RuBPCase activity and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (37). The purified RuBPCase had a specific activity 10-fold higher than that in the crude leaf extract and migrated as two bands with molecular weights of approximately 55,000 and 14,000 in SDS-PAGE.

The LSU and SSU polypeptides were separated by two methods. Approximately 7 mg of purified RuBPCase was subjected to SDS-PAGE in a 3-mm-thick, 20% polyacrylamide gel. The regions of the gel containing the LSU and SSU polypeptides were excised and homogenized with 40 mM Tris hydrochloride (pH 6.8)–1% SDS–10% (vol/vol) 2-mercaptoethanol, and the polypeptides were allowed to diffuse out of the gel for 24 h at 4°C. The gel pieces were removed by filtration through Whatman no. 1 filter paper, and the filtrates were dialyzed against three changes of 25 mM Tris hydrochloride (pH 6.8)–10 mM 2-mercaptoethanol at 4°C.

Alternatively, the two subunits were separated by gel filtration. Approximately 10 mg of purified RuBPCase was adjusted to 7 M urea in 80 mM phosphate buffer (pH 7.0), heated at 45°C for 15 min, and loaded onto a column (1.7 by 90 cm) of Sephadex G-75 equilibrated with 6 M urea in 80 mM phosphate buffer. Elution of LSU and SSU polypeptides was monitored by SDS-PAGE, and fractions containing the polypeptides were pooled separately and dialyzed against two changes of 80 mM phosphate buffer–10 mM 2-mercaptoethanol at 4°C.

Antiserum preparation. Antibodies to RuBPCase holoenzyme, the LSU and SSU polypeptides were raised in rabbits. Approximately 100 to 200 μg of each antigen was emulsified with Freund complete adjuvant and injected interdermally at six to eight sites on the back of each rabbit. After four weeks, each rabbit was given booster injections intramuscularly, twice at 2-week intervals, of 50 to 100 μg of antigen emulsified with Freund incomplete adjuvant. The specificity and titer of the antibody in the serum were monitored by ELISA (30, 71), immunoprecipitation assays, and Western immunoblotting.

Preparation of leaf extracts. Light- and dark-grown cotyledons were homogenized with 50 mM Tris hydrochloride (pH 7.2)–10 mM 2-mercaptoethanol–1 mM EDTA–10 μg of phenylmethylsulfonyl fluoride (PMSF) per ml in a Ten Broeck ground-glass homogenizer. The extract was centrifuged at 12,000 × *g* for 5 min, and the supernatant was retained. RuBPCase activity and protein were assayed immediately, and an additional sample was stored at −70°C in SDS-PAGE sample buffer (37) for further analysis.

Determination of LSU and SSU polypeptide levels. LSU and SSU levels were determined by an immunoblot technique. Extracts, each containing 1 μg of total protein, from light- and dark-grown cotyledons were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose paper electrophoretically for 18 h (26). After saturating nonspecific protein-binding sites on the nitrocellulose paper with bovine serum albumin, the paper was incubated first with 1% (vol/vol) antiserum to the LSU and SSU and then with ^{125}I-labeled protein A (69). Levels of LSU and SSU polypeptides were determined from an appropriately exposed film so that the signal was in the linear response of the film. Furthermore, standard amounts of purified RuBPCase were subjected to this procedure, and the levels of LSU and SSU in the experiment were kept within the linear range of detection of this methodology.

The autoradiograph shown in the text and those used for densitometry were obtained without intensifying screens so that band intensity would be proportional to the radioactivity on the blot. Densitometer tracings of the autoradiographs were performed with a Joyce-Loebl model 3CS microdensitometer, and areas under the peaks were determined with a Numonic Corp. model 1224 electronic graphics calculator.

In vivo labeling of proteins. Cotyledons were excised from the petiole under water to prevent entrapment of air in the vascular tissue. Cotyledons were then quickly transferred to a 200-μl solution of 100-μCi [^{35}S]methionine (1,000 Ci/mmol; Amersham Corp.). Cotyledons (3 to 10) were allowed to incorporate label for 2 h and were then homogenized with 0.5 ml of 50 mM Tris hydrochloride (pH 7.2)–10 mM 2-mercaptoethanol–1 mM EDTA–20 μg of PMSF per ml in a Ten Broeck ground-glass homogenizer. The extract was centrifuged at 12,000 × *g* for 5 min. To 0.4 ml of the supernatant was added 0.1 ml of 0.75 M NaCl–50 mM Tris hydrochloride (pH 7.4)–25 mM EDTA–5% sodium deoxycholate–0.5% SDS, followed by freezing at −70°C. Incorporation of [^{35}S]methionine into protein was determined as the trichloroacetic acid-insoluble radioactivity (42). [^{35}S]methionine-labeled LSU and SSU polypeptides were immunoprecipitated from samples containing equal radioactivity incorporated into protein. Extracts were adjusted to 0.5% SDS and heated at 60°C for 20 min. They were then

cooled to 4°C and adjusted to a final volume of 0.5 ml containing 0.15 M NaCl, 10 mM Tris hydrocholride (pH 7.4), 5 mM EDTA, 1% sodium deoxycholate, and 0.1% SDS (RIPA buffer). Ten microliters of each antiserum to LSU and SSU polypeptide was added and incubated at 4°C for 1 to 2 h. Then 100 μl of a 10% suspension of Formalin-treated *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring) in RIPA buffer was added and incubated at 4°C for an additional 30 min. The immunocomplexes were washed three times with 1-ml volumes of RIPA buffer and finally suspended in 30 μl of SDS-PAGE sample buffer (37), heated at 100°C for 5 min, and centrifuged at 12,000 × *g* for 5 min. The supernatant was subjected to SDS-PAGE in a 15% polyacrylamide gel, and the gel was fixed and fluorographed with sodium salicylate (16).

Cloning of LSU and SSU DNA sequences. Chloroplast DNA was isolated from amaranth leaves by the procedure of Bovenburg et al. (12). A 3.2-kilobase *Bam*HI fragment of amaranth chloroplast DNA containing the LSU gene was identified by using as a probe a ^{32}P-labeled, cloned fragment of maize chloroplast DNA (provided by J. Bedbrook) which encodes LSU (19). This 3.2-kilobase fragment was cloned into the *Bam*HI site of pBR322. This cloned plasmid, pAls1, has been used to detect the 1.6- and 1.8-kilobase LSU mRNAs of amaranth by Northern analysis of RNA and to direct the synthesis of the LSU polypeptide in an *Escherichia coli* cell-free, coupled transcription-translation system.

To obtain an SSU clone, polyadenylated RNA from amaranth leaves was size fractionated by sucrose density gradient centrifugation (5). Fractions enriched for the SSU mRNA were identified by in vitro translation in the rabbit reticulocyte cell-free system followed by immunoprecipitation with anti-SSU serum. Double-stranded cDNA was generated from this RNA by the method of Gubler and Hoffman (33), inserted into the *Pst*I site of pUC8 (70) by adding homopolymer tails (48), and used to transform *E. coli* TC600. The cDNA clones were analyzed by hybrid release translation (53) to detect those clones containing SSU DNA sequences. A plasmid, pAss1, was isolated from one of these clones and found to have an insert of approximately 750 base pairs. Details concerning the isolation and characterization of these clones will be described elsewhere.

Small-scale plasmid isolations for hybrid release translations were done by the procedure of Casse et al. (15). Large-scale plasmid isolations were done by the methods of Clewell and Helinski (18).

RNA extraction and Northern analysis. Approximately 1 g of cotyledons was frozen in liquid nitrogen and pulverized with a mortar and pestle. The powder was homogenized in 5 ml of 0°C 50 mM Tris hydrochloride (pH 8.0)–10 mM EDTA–2% SDS–0.1 M diethyldithiocarbonic acid–0.01% phenol for approximately 1 min with a Tekmar Tissumizer. The homogenate was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (50:50:1) and centrifuged at 2,000 × *g* for 15 min at 4°C. The phenol-chloroform-isoamyl-alcohol extraction was repeated once, and the RNA was adjusted to 0.3 M sodium acetate and precipitated with 2 volumes of ethanol overnight at −20°C. RNA was recovered by centrifugation, redissolved in 5 ml of sterile H_2O, and precipitated in 2 M LiCl for 4 h at 4°C to remove double-stranded nucleic acid (DNA). The LiCl-precipitated RNA was recovered by centrifugation at 12,000 × *g* for 30 min at 4°C, dissolved in H_2O, and precipitated in 0.3 M sodium acetate and ethanol. The RNA pellet was dissolved in H_2O and stored at −20°C. RNA was fractionated on agarose-formaldehyde gels and transferred to nitrocellulose paper for Northern analysis by the methods of Maniatis et al. (41).

In vitro translation. The functionality of LSU mRNA was tested by translation of total cellular RNA from amaranth in cell-free lysates prepared from *E. coli* by the method of Zubay (73). Similarly, functional SSU mRNA was detected by translating polyadenylated RNA from amaranth in nuclease-treated, cell-free rabbit reticulocyte lysates (51) incubated at 30°C for 60 min. Rabbit reticulocyte lysates were obtained from Green Hectares, Oregon, Wis.

Cell-free lysates were programmed with different amounts of each RNA preparation to determine the range of RNA concentrations which gave linear incorporation of [^{35}S]methionine. The amounts of RNA within the linear incorporation range were translated, and samples of in vitro-synthesized translation products containing equal amounts of trichloroacetic acid-insoluble radioactivity were used for immunoprecipitation with anti-LSU or -SSU serum as described above, except that incubation at 60°C was carried out for only 2 min. The immunocomplexes were subjected to SDS-PAGE and visualized by fluorography.

RESULTS

To precisely correlate changes in (i) enzyme activity, (ii) protein accumulation, (iii) protein synthesis, and (iv) mRNA levels, the data which are presented here represent the complete results of a single experiment. Five additional repeats of the experiment have been carried out, with the results in each case corresponding to those described below.

RuBPCase activity. RuBPCase specific activity in light- and dark-grown amaranth cotyledons was assayed 1 through 8 days postplanting (Fig. 1). In light-grown seedlings, RuBPCase activity was first detected at day 3 and increased

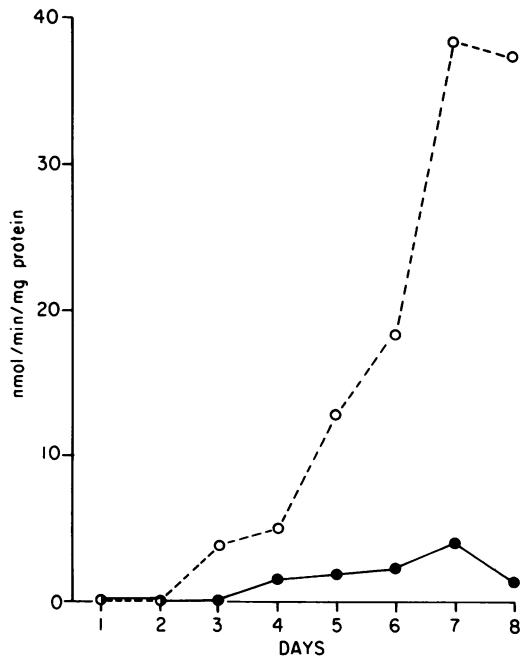

FIG. 1. RuBPCase-specific activity in light- (○) and dark-grown (●) cotyledons. Activity was determined in crude cotyledon extracts as $NaH^{14}CO_2$ fixation into acid-stable radioactivity.

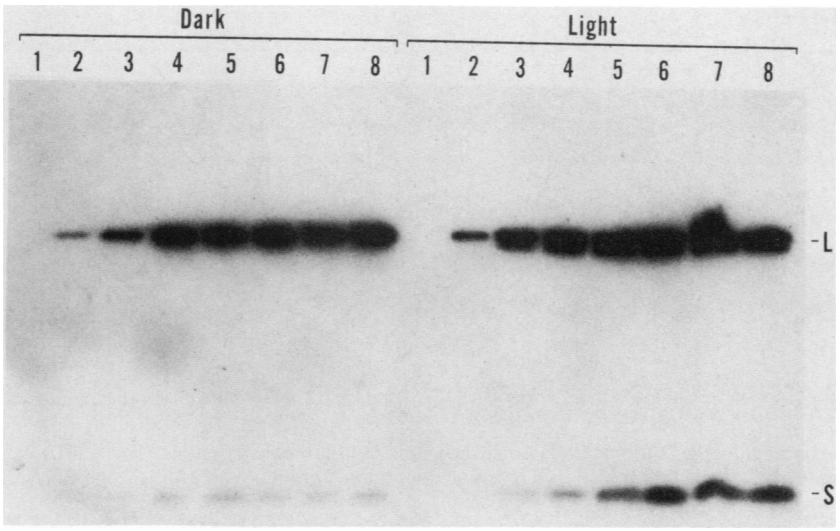

FIG. 2. LSU and SSU polypeptide accumulation in 1- through 8-day-old, dark- and light-grown cotyledons. Total-protein extracts were prepared from cotyledons, fractionated by SDS-PAGE, and blotted to nitrocellulose paper. Blots were probed with LSU and SSU polypeptide antiserum and subjected to autoradiography. The LSU doublet was due to proteolytic degradation of the polypeptide during storage.

10-fold by day 7, with activity leveling off by day 8. RuBPCase activity in dark-grown cotyledons was not detected until day 4 after planting and showed only a very slight increase to day 7. By day 8, activity was approximately 15-fold lower than that in cotyledons isolated from light-grown seedlings.

LSU and SSU accumulation. Immunoblot analysis of SDS-PAGE-fractionated proteins with antiserum against the LSU of amaranth RuBPCase showed that the levels of the LSU polypeptide increased during the growth of the cotyledons and were similar in light- and dark-grown seedlings (Fig. 2). Under both growth conditions, LSU was first detected on day 2, with accumulation reaching a plateau by day 5. Surprisingly, the observed changes in LSU levels did not correlate with the changes in enzyme activity shown in Fig. 1.

In contrast to the LSU, SSU protein levels were markedly different in light- and dark-grown cotyledons (Fig. 2). In light-grown cotyledons, SSU was first detected at day 3 (1 day later than the LSU) and increased 10- to 15-fold (as assayed by densitometry) by day 6. SSU protein in dark-grown cotyledons was not detected until day 4 (2 days after the LSU) and showed no significant increase through day 8. These results demonstrate that the accumulation of the LSU and SSU subunits was not coordinated in amaranth cotyledons.

The initial detection of RuBPCase activity in both light- and dark-grown seedlings corresponded to the initial appearance of the SSU protein (Fig. 1 and 2). Furthermore, changes in enzyme activity closely followed the levels of SSU detected by immunoblot analysis throughout the course of the experiment. Thus, in amaranth cotyledons RuBPCase activity in vivo appeared to be limited by the concentration of the SSU.

LSU and SSU synthesis in vivo. The in vivo synthesis of the LSU and SSU proteins was determined by using cotyledons which were detached from the plant and incubated in a solution containing [^{35}S]methionine. Proteins were extracted

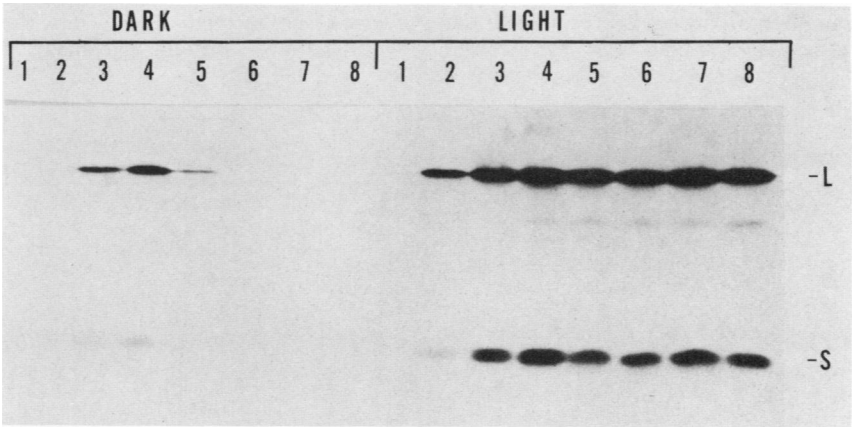

FIG. 3. In vivo synthesis of LSU and SSU polypeptides in 1- through 8-day-old, dark- and light-grown cotyledons. Seedlings were incubated with [^{35}S]methionine as described in the text. LSU and SSU polypeptides were immunoprecipitated from equal amounts of incorporated label, separated by SDS-PAGE, and fluorographed.

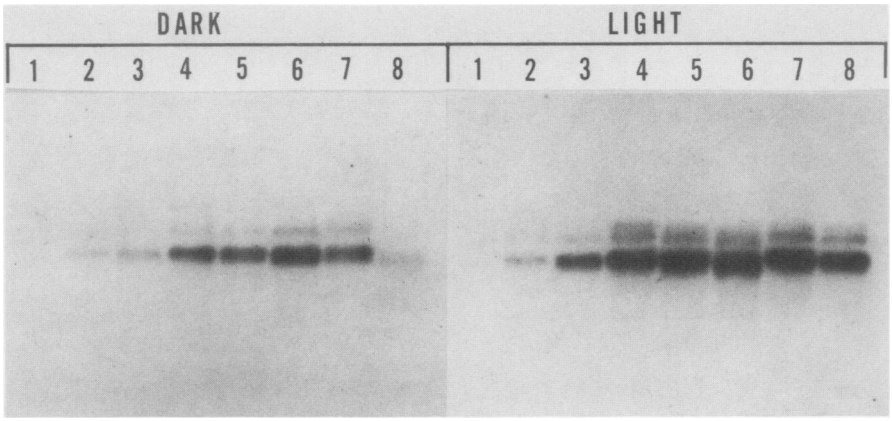

FIG. 4. LSU mRNA accumulation in 1- though 8-day-old, dark- and light-grown cotyledons. Total RNA extracted from cotyledons was fractionated on an agarose-formaldehyde gel, transferred to nitrocellulose, and probed with pAls1. Total RNA (5 μg) was used for each lane.

from the labeled cotyledons; LSU and SSU were immunoprecipitated from equal amounts of incorporated radioactivity, fractionated by SDS-PAGE, and fluorographed (Fig. 3). In light-grown cotyledons, LSU and SSU synthesis was initially observed at day 2 and continued throughout the growth of the cotyledons. Synthesis of both subunits reached a maximum by day 4 and continued through day 8. In contrast, in dark-grown cotyledons synthesis of both subunits occurred only as a burst between days 2 and 5 after sowing. Synthesis of both subunits in dark-grown cotyledons reached a maximum at day 4 and then declined sharply at day 5, with no detectable synthesis on days 6 through 8.

LSU and SSU mRNA. Northern analysis of total amaranth RNA with the pAls1 probe reveals two LSU mRNAs of 1.6 and 1.8 kilobases. Multiple LSU transcripts of similar sizes have been reported for several plant species (23, 47). LSU mRNA could first be detected in light-grown cotyledons on day 2 after sowing, with levels increasing and then plateauing on day 4 (Fig. 4). LSU mRNA in dark-grown cotyledons was also first observed on day 2; however, at peak levels (day 6) its concentration was two- to fivefold lower than in light-grown cotyledons. In dark-grown cotyledons the level of LSU mRNA decreased slightly by day 7 and dramatically by day 8.

LSU mRNA levels in light-grown cotyledons corresponded with observed synthesis of LSU protein on days 1 through 8 (Fig. 3 and 4). However, in dark-grown cotyledons, LSU mRNA accumulation did not correspond to the observed periods of synthesis for the LSU. LSU synthesis in etiolated seedlings stopped after day 5, even though LSU mRNA levels continued to increase through day 6 and did not significantly decline until day 8. These results suggest that LSU synthesis may have been post-transcriptionally blocked in dark-grown amaranth cotyledons starting on day 5.

SSU mRNA was detected by Northern analysis with the pAss1 amaranth SSU cDNA clone as a probe. SSU mRNA was present in both light- and dark-grown seedlings, with considerably higher levels occurring in the light-grown seedlings (Fig. 5). Low levels of SSU mRNA in light-grown cotyledons were observed on days 2 and 3, increased 20-fold (as assayed by densitometry) by day 4, and remained at this higher level through day 8. In dark-grown cotyledons SSU mRNA was first detected at low amounts on day 2, increased through day 4, and then dropped gradually through day 8. The amount of SSU mRNA in etiolated cotyledons was less than that in light-grown cotyledons at all of the time points, with a ≥20-fold difference on days 4 through 8.

SSU protein synthesis reflected SSU mRNA levels in light-grown seedlings. For example, an increase in SSU mRNA on day 4 was concomitant with an increase in SSU protein synthesis on that day (Fig. 3 and 5). However, as with LSU, SSU mRNA levels in dark-grown cotyledons did not correspond with the period of synthesis for the SSU protein. Synthesis of the SSU protein in the dark occurred only as a burst between days 2 and 5, although SSU mRNA was present through day 8. Thus, these data suggest that, as with the LSU protein, the major control of SSU protein

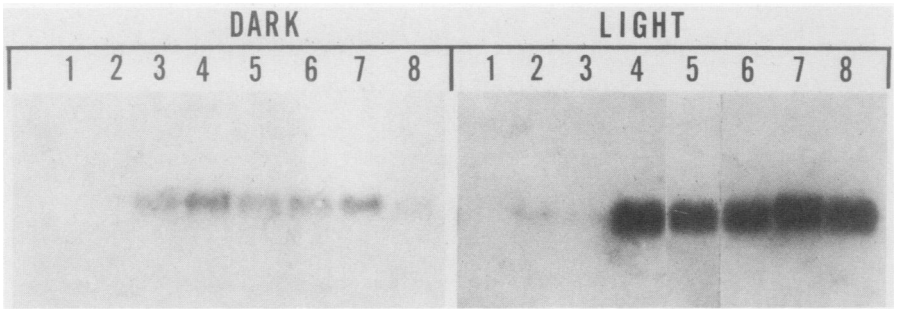

FIG. 5. SSU mRNA accumulation in 1- through 8-day-old, dark- and light-grown cotyledons. Total RNA, fractionated on an agarose-formaldehyde gel and transferred to nitrocellulose, was probed with pAss1. Total RNA (5 μg) was used for each lane.

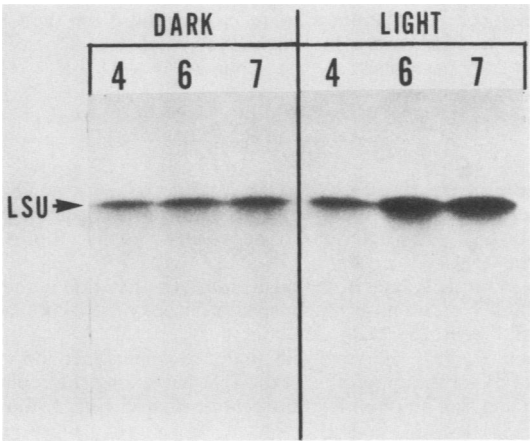

FIG. 6. Synthesis of LSU protein in vitro. Total RNAs isolated from 4-, 6-, and 7-day-old, dark- and light-grown cotyledons were used to program an *E. coli*, cell-free translation system. LSU was immunoprecipitated from the reactions, subjected to SDS-PAGE, and fluorographed.

synthesis in dark-grown amaranth cotyledons after day 5 was exercised at the post-transcriptional level.

Functionality of LSU and SSU mRNA. The functionality of the LSU mRNA was tested with an in vitro translation system derived from *E. coli*, since chloroplast genes, RNAs, and ribosomes have procaryotic rather than eucaryotic features. The in vitro synthesized LSU protein was immunoprecipitated from the reactions by using anti-LSU serum and examined by SDS-PAGE. The LSU mRNA isolated from 6- and 7-day-old, dark-grown cotyledons (when synthesis was not observed in vivo) was as translatable in vitro as mRNA prepared from 4-day-old, dark-grown seedlings (when synthesis was occurring in vivo) (Fig. 6). The amount of LSU produced from both light- and dark-grown cotyledon mRNA in vitro correlated with the amounts of LSU mRNA determined by Northern analysis, rather than with the amount of protein produced in vivo. Therefore, the block to LSU polypeptide synthesis in dark-grown cotyledons in vivo after day 4 was not due to an alteration in the translatability of the LSU mRNA.

The functionality of SSU mRNA was examined with a rabbit reticulocyte, cell-free translation system. Immunoprecipitation with amaranth anti-SSU serum and analysis by SDS-PAGE revealed that the 20-kDa precursor to the 14-kDa mature SSU protein was produced when amaranth polyadenylated mRNA was used in the in vitro system. Moderate levels of SSU were produced when the reticulocyte system was programmed with mRNA from 5- (when synthesis in vivo sharply declined) and 7-day-old (when synthesis in vivo had ceased), dark-grown cotyledons (Fig. 7). These levels were similar to those produced with mRNAs isolated from etiolated seedlings showing peak in vivo synthesis (days 3 and 4). As with LSU, SSU produced in vitro in both light- and dark-grown seedlings generally reflected the amounts of SSU mRNA detected by Northern analysis. Thus, as with LSU, the block to SSU gene expression in dark-grown cotyledons after day 4 was not due to changes in RNA functionality.

DISCUSSION

The expression of the genes encoding RuBPCase in a variety of plant species has been under intensive study. The effect of light on RuBPCase production has been shown to vary among these different plant groups. We present here a study of the expression of RuBPCase genes in *A. hypochondriacus*, a dicotyledonous plant which utilizes the C_4 photosynthetic pathway. This unique subclass of plants has not previously been examined with respect to RuBPCase gene expression. In amaranth, the light-mediated regulation of RuBPCase production was complex. The degree of light-induced expression observed was dependent on the time postplanting at which the analyses were done as well as on the methods used for analyses. This was due to the presence of several levels of control which regulated the production of RuBPCase in the developing seedlings. This complex transcriptional and post-transcriptional control accounted for the final accumulation of the LSU and SSU polypeptides and therefore RuBPCase activity in light- and dark-grown amaranth seedlings.

RuBPCase specific activity in light-grown cotyledons increased rapidly during growth to a level 15-fold higher than that in dark-grown cotyledons by day 8 after planting. Although the levels of the LSU polypeptide were quite similar in light- and dark-grown cotyledons, the levels of SSU closely paralleled changes in enzyme activity. These results suggest that the level of SSU controls the amount of enzyme activity and are consistent with studies which have demonstrated that, although the substrate binding site is located on the LSU, the SSU is essential for enzyme catalysis (1, 2, 62, 63). Our results extended this data by demonstrating in vivo the essentiality of the SSU for RuBPCase activity.

Studies of a variety of C_3 and C_4 monocots have indicated that the accumulation of LSU and SSU is tightly coordinated in developing leaves (24, 49, 50). Preliminary evidence from our laboratory also indicates that accumulation of the two subunits is coordinated in leaves of the C_4 dicot amaranth. In contrast, we found that accumulation of LSU and SSU is not coordinate in amaranth cotyledons, suggesting that these two tissues (leaves and cotyledons) may regulate the expression of these genes quite differently.

Analysis of protein synthesis revealed that in light-grown seedlings the production of LSU and SSU polypeptides began on day 2 and continued during the growth of the cotyledons. In contrast, in dark-grown cotyledons, synthesis of both subunits occurred only as a burst between the days 2 and 5 after sowing. Therefore, light was not required to initiate either LSU or SSU mRNA or protein synthesis in

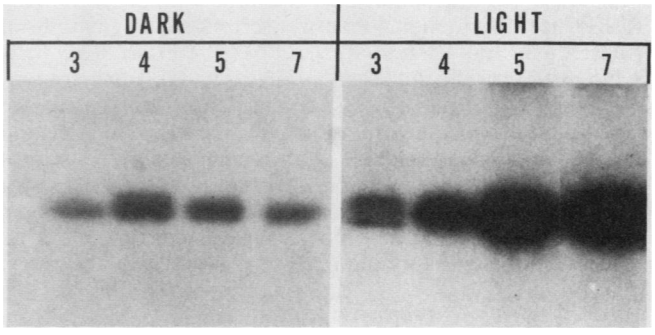

FIG. 7. Synthesis of SSU precursor in vitro. Polyadenylated mRNAs from 3-, 4-, 5-, and 7-day-old, dark- and light-grown cotyledons were used to program a rabbit reticulocyte, cell-free translation system. SSU precursor was immunoprecipitated from the reactions, subjected to SDS-PAGE, and fluorographed.

amaranth seedlings. Rather, it appeared that developmental signals were involved in this initiation event, whereas light was required for the continuation of RuBPCase synthesis on and after day 5.

mRNA levels for both LSU and SSU polypeptides were lower in dark-grown seedlings than in light-grown ones, indicating that the transcription or stability (or both) of these mRNAs was at least partially light-dependent. It is also possible that higher levels of LSU mRNA in light-grown seedlings were due to an increased LSU gene dosage, as has been reported for peas. (56).

In dark-grown seedlings synthesis of both subunits was not observed after day 5, even though the corresponding steady-state levels of the mRNAs remained similar in vivo and were translatable in vitro through day 7. Therefore, either there was a translational block to the synthesis of these two proteins or the newly synthesized polypeptides were very unstable in dark-grown cotyledons during and after day 5. The fact that both LSU and SSU levels remained constant on days 4 through 8 suggests that there was not rapid degradation of either subunit in the dark. We have also observed dramatic changes in the synthesis of LSU and SSU polypeptides within the first few hours of shifting light-grown cotyledons into darkness or dark-grown cotyledons into light, without any concomitant changes in mRNA levels (manuscript in preparation). These studies further support the conclusion that post-transcriptional regulation, in addition to transcriptional control, plays an important role in modulating the expression of these genes.

The post-transcriptional block described here may be specific to a small subset of polypeptides which include the LSU and SSU polypeptides. While [^{35}S]methionine incorporation into total proteins (as assayed by trichloroacetic acid-precipitable radioactivity and based on the intensity of the majority of bands analyzed by SDS-PAGE) was reduced approximately 10-fold in dark-grown seedlings, synthesis of SSU and LSU polypeptides, specifically, was diminished 100-fold or more (i.e., was undetectable). However, since the chloroplast-encoded polypeptides, not including LSU polypeptides, are not readily detectable by one-dimensional SDS-PAGE, we do not know whether translation by 70S chloroplastic ribosomes is more dramatically reduced than total protein synthesis in dark-grown seedlings. If this is the case, then the apparent specific block to LSU production could be due to a more generalized cessation of chloroplastic-protein synthesis. This aspect of protein synthesis regulation is currently under investigation.

Other studies have also provided evidence that post-transcriptional regulation may be involved in modulating RuBPCase production. Post-transcriptional regulation of RuBPCase has been reported for *Chlamydomonas reinhardtii* (45) and for *Euglena gracilis* (44). Furthermore, post-transcriptional control, in addition to control at the level of mRNA synthesis or turnover, has been documented for another nuclear-encoded chloroplast protein, the chlorophyll a/b-binding protein (8, 59, 61, 66, 67). Thus, our own results and those of others suggest that post-transcriptional regulation, in addition to transcriptional control, plays an important role in determining the expression of plant genes.

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